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REPORT 86.48 (APS 2) Pr.nr. 505.0720

Subject: Introduction of the concept "Adequate Peaks Search" for confirmation of the presence of an analyte in a sample by infrared spectroscopy.

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REPORT 86.48 (APS 2) Pr.nr. 505.0720

Project: Development of spectroscopic methods of analysis.
Subject: Introduction of the concept "Adequate Peaks Search" for confirmation of the presence of an analyte in a sample by infrared spectroscopy.
(APS series, part 2).

Preceeding report 86.48 (APS 1).

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Aim:
To present unambiguous, objective criteria for the confirmation of the presence of an analyte in a sample by infrared spectrometry.

Summary:
Due to the large information density of the measuring result, spectroscopic techniques are in particular suitable to confirm the presence of a substance.
However, until now, infrared spectra could hardly be interpreted in an unambiguous, objective way.
To overcome this drawback, the concept "Adequate Peaks" is introduced.
With help of adequate peaks tables a spectrum search can be carried out, resulting in a "score".
Criteria for the use of the "Adequate Peaks Search" method for making a decision about the confirmation of an analyte in a sample are given.
An example of an Adequate Peaks Search is presented.

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Projectleader: J.M. Weseman

## 1. Introduction

In analytical chemistry for control purposes, and especially in forensic investigations, there is a need for an unambiguous decision about the presence or absence of a searched analyte in a sample under investigation.

Therefore it is recommended, when an analyte is positively detected by an analytical method (the "screening method"), to confirm this result by a second method based upon another measuring principle (the "confirmation method").

For confirmation, spectroscopic techniques are in particular suitable due to the large information density of the measuring result, i.e. the resulting spectra.

Although infrared spectroscopy even surpasses other spectroscopic techniques in this respect, one drawback was, that an infrared spectrum could hardly be described in objective terms.

Judging infrared spectra by infrared experts is normally carried out by considering characteristic bands and further by an overview of the whole spectrum, and the conclusion is based on a subjective opinion.

In forensic control, unlike in production control, the aim is not to demonstrate whether or not two substances are identical, but to prove that a distinct chemical compound is present in an investigated sample - even in the case that there are obviously differences between the spectra of sample and of standard material.

Therefore there is a need for objective criteria for interpretation of infrared spectroscopic results, which can be verified without subjective considerations.

In this report such objective, digitalized criteria are presented, using so-called "Adequate Peaks", and applied to reference infrared spectra of anabolics and related compounds.

## 2. Definition of adequate peaks

Adequate peaks are absorption maxima in the infrared spectrum of a standard material, that fulfil the following requirements.

2.1 The absorption maximum is in the wavenumber range 1800-500 $cm^{-1}$.

2.2 The intensity of the absorption is not less than:

2.2.1 a specific molar absorbance coefficient

- of 40 with respect to zero absorbance and
- of 20 with respect to the peak base line

or

2.2.2 a relative absorbance

- of 12.5% of the absorbance the most intense peak in the region 1800-500 $cm^{-1}$, when both peaks are measured with respect to zero absorbance and
- of 5% of the absorbance of the most intense peak in the region 1800-500 $cm^{-1}$, when both peaks are measured with respect to their peak base line.

NOTE: Although adequate peaks according to 2.2.1 may be preferred from a theoretical point of view, those according to 2.2.2 are easier to determine in practice.

The exact peak positions of the adequate peaks, in whole wavenumbers, act as objective, digitalized parameters for judging spectra of samples.

3. Definition of "score".

The "score" is the percentage of the adequate peaks of the standard material, that is found in the infrared spectrum of the analyte.

4. Use of the adequate peak tables

4.1 The positions of the peaks in the infrared spectrum of the analyte are compared with the positions of the adequate peaks in the infrared spectrum of the standard material.

4.2 The number of peaks in the infrared spectrum of the analyte whose frequencies correspond with adequate peaks in the infrared spectrum of the standard material, within a margin of $\pm$ 1 $cm^{-1}$, is determined.

A minimum of 6 adequate peaks in the IR spectrum of the standard material is required. If less than 6 adequate peaks are present in the spectrum of a compound the presence of this compound cannot be proved by this approach.

4.3 The "score" of the standard material in the analyte spectrum is calculated.

5. Criteria

5.1 The score of the standard material shall be at least 50%.

5.2 Where no match is found in the sample spectrum for an adequate peak of the standard material, the sample spectrum shall not exclude the possibility of the presence of the absorption corresponding with the adequate peak (see figure 1).

5.3 The procedure is only applicable to absorption peaks in the sample spectrum with an intensity of at least 3 times the peak to peak noise.

6. Use of the criteria

6.1 The presence of the analyte in the test sample is proved when all the criteria* are fulfilled.

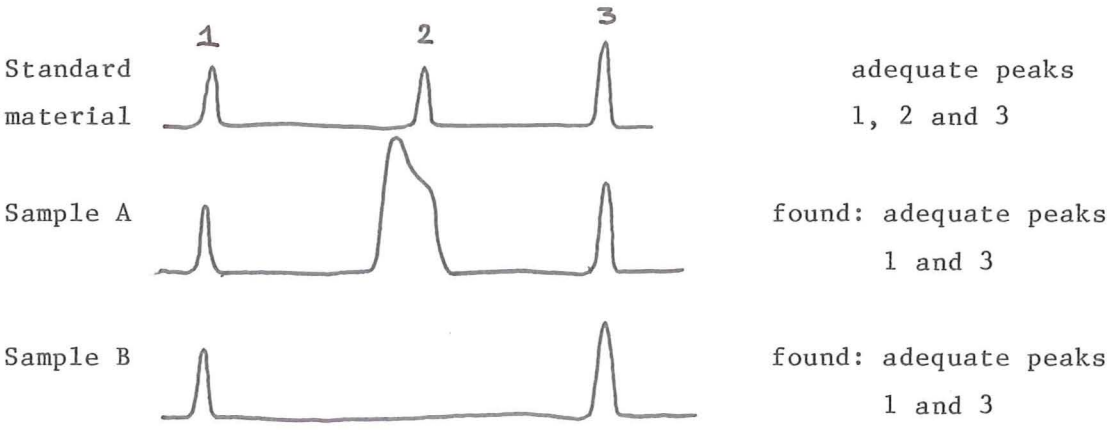
6.2 If not all criteria* are fulfilled, the presence of the analyte in the test sample is not proved.

6.3 The analysis does not decide about the absence of the analyte in the test sample.

6.4 If less than 6 adequate peaks are found in the IR spectrum of the standard material, the method is not applicable.

*Note. This includes also criteria which may be stated for pretreatment of the test sample prior to the infrared detection, not discussed here.

Figure 1: Illustration of criterion 5.2




The spectrum of sample A does not exclude the presence of adequate peak 2 --> criterion 5.2 is fulfilled.
The spectrum of sample B excludes the presence of adequate peak 2 --> criterion 5.2 is not fulfilled.

7. Example

Spectrum 1 is the infrared spectrum of a standard material, in this case 17β-methyltestosterone, an anabolic substance.
Spectrum 2 represents the infrared spectrum made from a sample in which the presence of anabolics has to be proved. The sample, a piece of meat, was extracted, the extract was cleaned up and a fraction in which anabolics were expected was isolated by HPLC. After evaporation of the mobile phase, a diffuse reflection spectrum of this fraction in KBr was recorded.

The Adequate Peaks Search proceeds as follows.

7.1 Determination of the Adequate Peaks in the infrared spectrum of the standard material (Spectrum 1).

7.1.1 In the region 1800-500 $cm^{-1}$ of this spectrum the strongest absortion is at 1664 $cm^{-1}$.

7.1.2 The absorption bands in the region 1800-500 $cm^{-1}$ having an absorbance of > 12.5% of the absorbance of the peak at 1664 $cm^{-1}$, both with respect to zero absorption, are selected.
These bands are listed in Table 1, column (a), their relative intensity is listed in column (b).

7.1.3 From these peaks it is checked whether their absorbance with respect to their own base line is > 5% than the absorbance of the maximum peak at 1664 $cm^{-1}$, see Table 1(c).

7.1.4 The bands fulfilling both requirements are the "Adequate Peaks". These peaks are listed in Table 1(d) and marked by in Spectrum 1.

7.1.5 The number of Adequate Peaks is 12, also more than 6, so this infrared spectrum is suitable for application of the Adequate Peaks Search.

7.2. Application of the Adequate Peaks Search on the sample spectrum (Spectrum 2).

7.2.1 The exact wavenumbers of the peaks in the sample spectrum are determined.

7.2.2 From the list of peak positions, thus obtained, the peaks with the same wavenumber $\pm$ 1 $cm^{-1}$ as the adequate peaks in the reference spectrum 1 are selected. These peaks are listed in Table 2(b), together with the adequate peaks of the reference spectrum (Table 2(a))

7.2.3 The intensities of these peaks are higher than 3 times the peak to peak noise.

7.2.4 In the sample spectrum 9 out of 12 adequate peaks of the reference spectrum are found by the computer program, i.e. 75%, i.e. more than the 50% required in the criteria.

7.2.5 Not found by the computer program in the sample spectrum are the following adequate peaks of the reference spectrum: 1611, 1450 and 1278 $cm^{-1}$. The presence of these bands in Spectrum 2 is obvious. The explanation is given in Table 2(a).

7.3 Conclusion

The Adequate Peaks Search fulfils all requirements of the criteria. The presence of 17β-methyltestosterone in the sample investigated is confirmed.

8. Validation

The Adequate Peaks Search method has been accepted as a reliable method for the detection of analytes in test samples by a group of experts on infrared spectroscopy composed of G. Dijkstra, chairman (RIVM), L.A. van Dijck (Organon), C. Funcke (Organon), J.H. van der Maas (RUU), W.G. de Ruig (RIKILT, W.A. Seth Paul (Jassen Pharmaceutica) and R.W. Stephany (RIVM) on 1987-03-19.*

It is recommended that, when comparing the infrared spectra of standard material and sample, the standard material is treated according to the same entire procedure as the sample, and the spectra are run under the same conditions. If this requirement is not fulfilled, that may give rise to falls negative results, i.e. the analyte is present, but not detected; however, it will not cause falls positive results, i.e. the analyte is absent, but nevertheless detected as being present.

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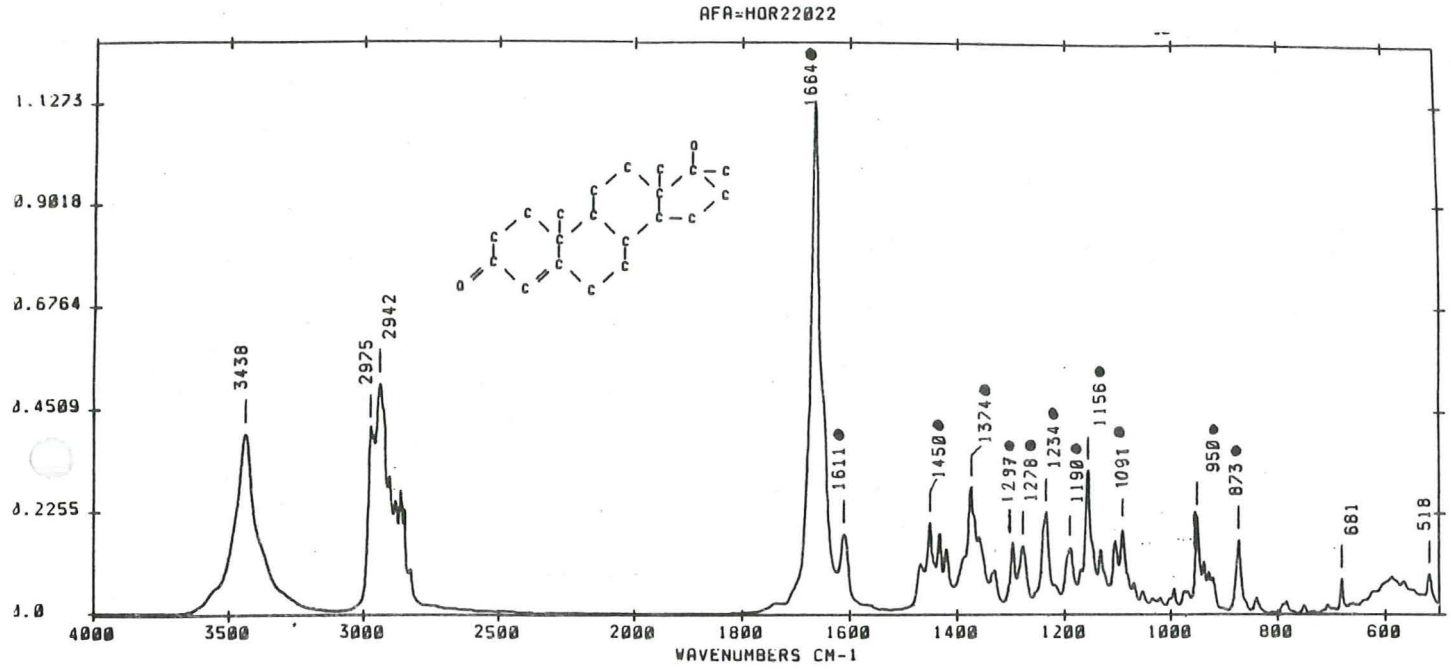
  Organon International B.V., Oss, The Netherlands
  RIKILT = State Institute for Quality Control of Agricultural Products, Wageningen, The Netherlands
  RIVM = National Institute of Public Health and Environment Hygiëne, Bilthoven, The Netherlands
  RUU = University of Utrecht, Utrecht, The Netherlands.

Spectrum 1: Reference spectrum of standard material 17α-methyl testosterone.




Spectrum 2: Spectrum of sample.

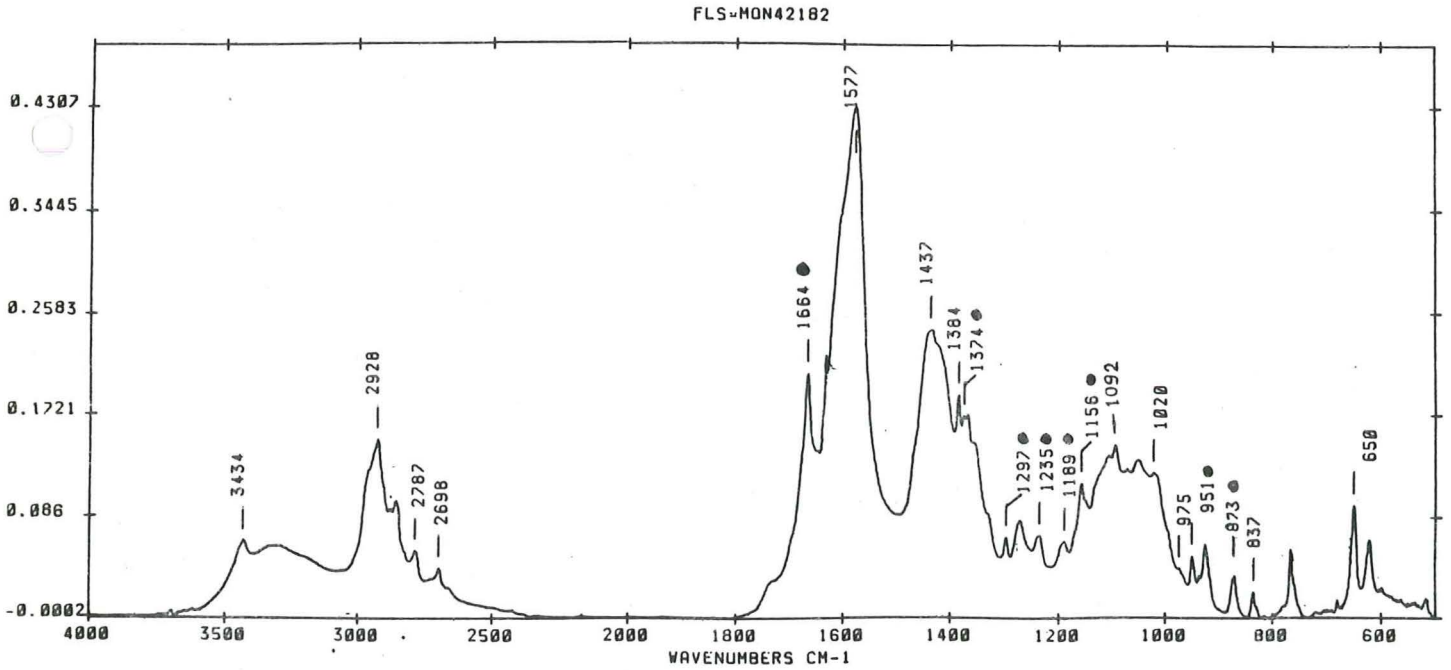



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Table 1: Determination of adequate peaks.

Standard material: 17β-methyltestosterone

| Wavenumber (a) | Rel. intensity zero obs. (b) | Rel. intensity with respect to base line > 5% (c) | Adequate Peaks (d) |
|---|---|---|---|
| cm-1 | % | | cm-1 |
| 1664 | 100 | yes | 1664 |
| 1611 | 16 | yes | 1611 |
| 1450 | 18 | yes | 1450 |
| 1432 | 16 | no | |
| 1420 | 13 | no | |
| 1374 | 25 | yes | 1374 |
| 1360 | 15 | no | |
| 1297 | 14 | yes | 1297 |
| 1278 | 14 | yes | 1278 |
| 1234 | 20 | yes | 1234 |
| 1190 | 13 | yes | 1190 |
| 1156 | 28 | yes | 1156 |
| 1133 | 13 | no | |
| 1105 | 14 | no | |
| 1091 | 17 | yes | 1091 |
| 950 | 19 | yes | 950 |
| 873 | 15 | yes | 873 |

Number of adequate peaks: 12.

Table 2: Adequate peaks of methyltestosterone searched in sample spectrum.

| Adequate peaks 17β-methyl-testosterone (a) | Found in sample spectrum (b) | Reason not found in sample spectrum (c) |
|---|---|---|
| cm-1 | cm-1 | |
| 1664 | 1664 | |
| 1611 | | present as shoulder on band of 1577 |
| 1450 | | present as broadening of band at 1437 |
| 1374 | 1374 | |
| 1297 | 1297 | |
| 1278 | | broadening of band at 1271 |
| 1234 | 1235 | |
| 1190 | 1189 | |
| 1156 | 1156 | |
| 1091 | 1092 | |
| 950 | 951 | |
| 873 | 873 | |